THE LIMITING RATE OF CHELATION OF LIVER ALCOHOL DEHYDROGENASE †

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Summary. The binding rates of two zinc-chelators, bipyridyl and pyrazole, to liver alcohol dehydrogenase have been studied by stopped-flow methods. The pseudo first-order rate constant for binding bipyridyl to liver alcohol dehydrogenase is 228 sec^{-1} at extrapolated infinite concentrations, while that for binding pyrazole to enzyme-NAD^+ complex is 238 sec^{-1}. From these data it is postulated that any coenzyme or substrate which binds to the zinc of LADH cannot be bound at a rate greater than 230 sec^{-1}.

The role of zinc in LADH* has been studied by a variety of methods, including conventional spectrophotometry, optical rotatory dispersion, circular dichroism and steady-state kinetics, none of which has yielded conclusive evidence for the actual function of zinc. Several zinc-chelating agents have been reported to be effective inhibitors of LADH (1,2). Some of these chelators, such as phenanthroline (1,3) and bipyridyl (1,4), are inhibitors competitive with both coenzyme and substrate, while others, such as pyrazole (5), compete only with substrate. In both instances, chelation to the zinc ion at the active site of LADH is the assumed mode of binding.

Steady-state kinetic evidence for a binary enzyme-bipyridyl complex has been presented by Plane and Theorell (1); direct spectrophotometric demonstration of such a complex has been re-

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* Abbreviations: LADH, liver alcohol dehydrogenase; bipyridyl, 2,2'-dipyridyl; phenanthroline, 1,10-phenanthroline.

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ported by Sigman (4), who also reported steady-state kinetic data in support of formation of a small amount of ternary complex. In contrast pyrazole exhibits only a slight tendency toward binary complex formation, but is tightly bound in a ternary complex (6). Dalziel (7) has concluded that the steady-state kinetics of phenanthroline inhibition indicate that the chelation is competitive with coenzymes but that it is not possible to ascertain whether substrates are bound to zinc using steady-state techniques. The fact that chelating agents bind to the enzyme and cause competitive inhibition does not preclude an hypothesis that zinc is merely near the active site and not involved in binding or catalysis.

Recently, several investigators have studied the role of zinc in LADH. Hoagstrom, et. al., (8) demonstrated that NADH can bind to zinc-free apoenzyme, and Drum (9) has replaced the zinc of LADH with cobalt and cadmium. The present study reports a stopped-flow investigation of the interaction of LADH with chelating agents in an attempt to define the role of zinc in the binding of coenzymes and substrates.

Methods. Crystalline alcohol dehydrogenase was prepared from horse liver by the method of Theorell et. al. (10). The concentration of enzyme was determined by the assay method of Dalziel (11) based on A_{280} of 0.455 ml mg^{-1} cm^{-1} and by titration with NADH in the presence of isobutyramide (12). NAD^+ was purchased from Sigma Chemical Company, bipyridyl from Fisher Scientific Company, and pyrazole from Eastman Chemical Company.

Difference spectra were obtained by the method of Theorell and Yonetani (13) using a Cary Model 11 spectrophotometer. All experiments were performed in sodium phosphate, 0.1 ionic strength, pH 7, at 24°C. Determination of the dissociation constant of bipyridyl from LADH was performed by the method of Sigman (4).

Stopped-flow studies were performed with a Durrum-Gibson instrument at 308 mμ for bipyridyl and 300 mμ for pyrazole. The time constant was 0.1 msec, the instrument dead-time was 2 msec, and the cuvette path length was 2.0 cm. The slit widths used were 1 mm. Results were plotted as pseudo first order reactions, and the rate constants were calculated from the slopes.

Results and Discussion. Stopped-flow measurements were made of the binding rate of bipyridyl to LADH at 308 mμ, the wave length of maximum absorbance for the binary complex as determined by difference spectrophotometry. The pseudo first-order rate constants were calculated using the equation for a reversible first order reaction (14), on the basis of a binary complex dissociation constant of 350 μM, determined by spectrophotometric titration. A plot of reciprocal pseudo first-order rate constants vs. reciprocal bipyridyl concentrations (Figure 1) resulted in a rate constant of 228 sec^{-1} at extrapolated infinite bipyridyl concentrations.

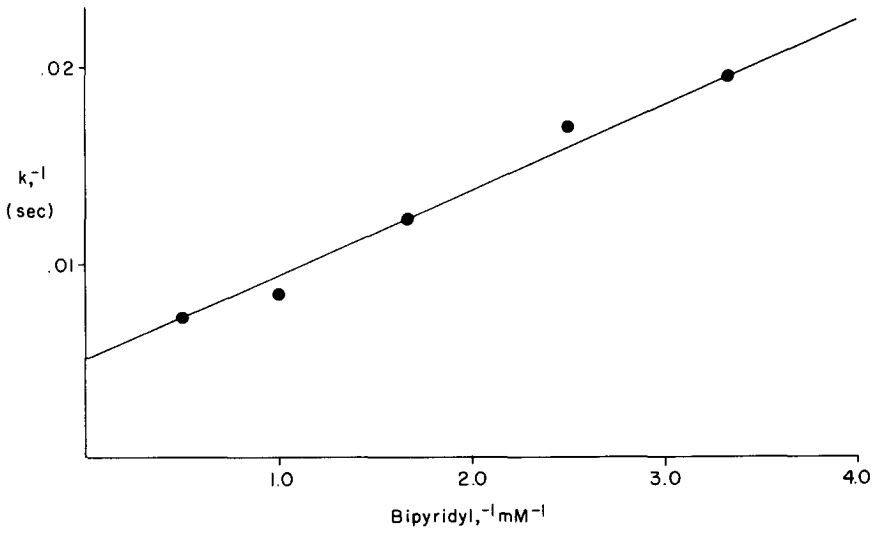

Figure 1. Double-reciprocal plot of pseudo first-order rate constants and bipyridyl in sodium phosphate, 0.1 ionic strength, pH 7, 24°C. The concentration of LADH after mixing was 11.9 μN.

Stopped-flow measurements of the binding rate of pyrazole to LADH-NAD^+ complex were made at 300 mμ, a wave length convenient for measuring formation of the ternary complex yet far from NAD^+ absorbance. Saturating concentrations of NAD^+ were used in order to convert all of the enzyme to binary complex. Since pyrazole is very tightly bound to the binary complex, pseudo first-order rate constants were calculated for several pyrazole concentrations using the equation for an irreversible first-order reaction (14). A plot of reciprocal pseudo first-order rate constants vs. reciprocal pyrazole concentrations (Figure 2) resulted in a rate constant of 238 sec^{-1} at extrapolated infinite pyrazole concentration.

Since most chelation reactions are slower than would be expected for diffusion-limited rates, it is believed that chelation consists of two steps (15), the first being the formation of an outer-sphere complex in a rapid equilibrium, and the second and rate-determining step being the loss of a solvent molecule from

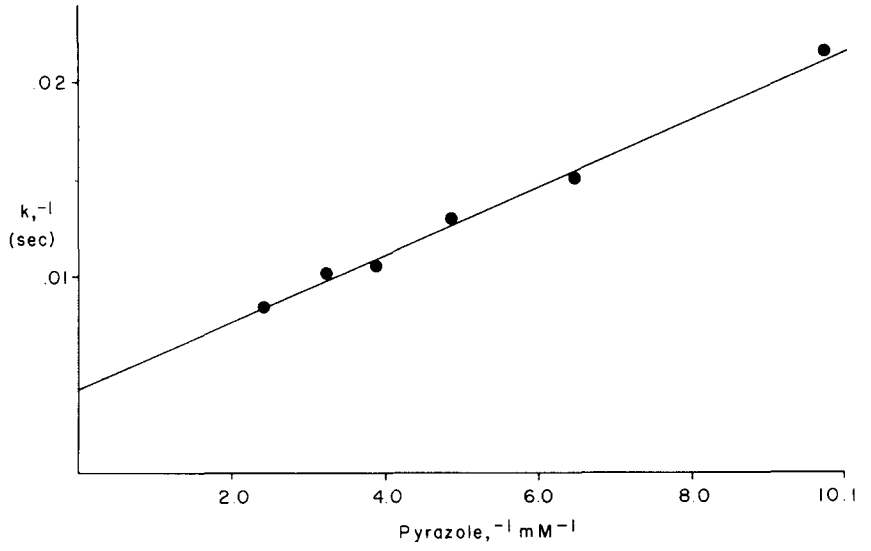

Figure 2. Double-reciprocal plot of pseudo first-order rate constants for the LADH-NAD^+ complex plus pyrazole reaction in sodium phosphate, 0.1 ionic strength, pH 7, 24°C. Concentrations after mixing were as follows: LADH, 5.0 μN; NAD^+, 2.6 mM.

the first hydration sphere of the metal ion. The present study indicates a rate-limiting step in chelation of the zinc of LADH, and by analogy it would seem that this step is displacement of a water molecule from the first hydration sphere of zinc. The limiting rate constant of 228 sec^{-1} represents the dissociation rate of water from the LADH-bound zinc ion, and the binding of any molecule to the enzyme at a faster rate cannot be due to metal chelation as the primary association factor. The fact that pyrazole-binding exhibits this rate-limiting step indicates not only that LADH-NAD^+-pyrazole complex involves zinc but, since pyrazole reacts with the pre-formed LADH-NAD^+ complex, that the binding of NAD^+ to LADH does not involve zinc. The work of Geraci and Gibson (15) on the binding of NADH to LADH did not extrapolate to a limiting pseudo first-order rate constant approximating 228 sec^{-1}, indicating that zinc is probably not involved in the binding of reduced coenzyme. The value of 228 sec^{-1} is offered as a kinetic criterion for determining whether the binding of coenzymes, substrates and inhibitors to LADH involves zinc. It is anticipated that this value will provide a valuable tool in evaluating the function of zinc in the LADH mechanism, and that the approach presented in this study will be generally applicable to other metallo-enzymes.

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